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14. ABSTRACT

Mantle cell lymphoma (MCL) is an aggressive B-cell malignancy characterized by over-expression of the cell cycle regulatory protein cyclin D1 (CCND1). Increased CCND1 expression levels are strongly associated with shorter patient survival related to chemoresistance, but little is known about the contribution of CCND1 to the resistant nature of MCL. On the basis of our preliminary data, we *hypothesize* that cyclin D1 plays a genome protective role through regulating expression of *CDK5RAP3* (C53), which encodes a putative tumor suppressor known to antagonize the DNA checkpoint kinase CHEK1. We test this hypothesis by focusing on the following *specific aims*: 1) determine the effect of C53 depletion or overexpression on CCND1 knockdown phenotypes; 2) determine the role of CCND1 in regulating C53 expression. This report describes results from specific aim 1. We have found that C53 depletion reduces DNA damage and restores ATR signaling in CCND1-depleted MCL cells, consistent with the rescue of CCND1/C53 double-knockdown cells and suggesting a role for C53 in mediating cytotoxicity of CCND1 knockdown. However, overexpression of C53 isoforms in the MCL cell line UPN-1 did not recapitulate CCND1 KD phenotypes as anticipated. We have proposed alternative approaches to address these discordant results.

15. SUBJECT TERMS
Mantle cell lymphoma, cyclin D1, chemoresistance, CDK5RAP3

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1 INTRODUCTION:

Mantle cell lymphoma (MCL) is an aggressive B-cell malignancy characterized by over-expression of the cell cycle regulatory protein cyclin D1 (CCND1). Increased CCND1 expression levels are strongly associated with shorter patient survival related to chemoresistance, but little is known about the contribution of CCND1 to the resistant nature of MCL. In preliminary studies, we found that depletion of CCND1 increased DNA damage and sensitized MCL cells to chemotherapeutic agents. We also found that CCND1 depletion led to increased transcription of *CDK5RAP3* and cytotoxicity of CCND1-depleted cells can be rescued by CDK5RAP3 depletion. *CDK5RAP3* encodes a putative tumor suppressor known to antagonize the DNA checkpoint kinase CHEK1. The **goal** of the project is to elucidate the genome-protective role of CCND1 through regulation of CDK5RAP3 expression. To accomplish this goal, we will focus on the following **specific aims**: 1) determine the effect of CDK5RAP3 depletion or overexpression on CCND1 knockdown phenotypes; 2) determine the role of CCND1 in regulating CDK5RAP3 expression.

2 KEYWORDS: Mantle cell lymphoma, cyclin D1, chemoresistance, DNA damage, CDK5RAP3, CHEK1

3 ACCOMPLISHMENTS:
What were the major goals of the project?

As stated in the SOW, the major goals of the project within this reporting period are to determine the effect of CDK5RAP3 (C53) depletion or overexpression on CCND1 knockdown (KD) phenotypes. Specifically, we will:

1. Determine the effect of C53 depletion on CCND1 KD phenotypes (months 1-6).
- Determine if C53 inactivation rescues spontaneous DNA damage associated with CCND1 silencing, using immunofluorescence staining for nuclear gamma-H2AX foci in CCND1 KD and CCND1/C53 DKD cells.
- Determine if C53 inactivation rescues defective ATR and CHEK1/2 signaling in CCND1-silenced cells exposed to DNA replication inhibitors (hydroxyurea or aphidicolin) or DNA damaging agents (etoposide or irradiation).

2. Determine whether C53 overexpression phenocopies CCND1 KD (months 6-12).
- Overexpress FLAG-tagged C53 isoform B or E in UPN-1 cells and determine whether these isoforms inhibit activity of CHEK1 or CHEK2, respectively.
- Determine whether overexpression of C53 isoforms is sufficient to induce apoptosis in unperturbed cells or sensitizes them to chemotherapeutic agents.
- If overexpression of isoform E does not induce apoptosis or increase sensitivity to cytotoxic agents, we will repeat these experiments in the context of CCND1 knockdown or test additional isoforms of C53.

Figure 1. Verification of mRNA knockdown. UPN-1 cells were transduced with Dox-inducible CCND1 or both CCND1 and C53 shRNA and selected with puromycin. mRNAs were prepared after 4-day Dox induction and analyzed by quantitative PCR (qPCR). Shown are means of three independent experiments. Error bars, SD.

What was accomplished under these goals?

C53 depletion reduces DNA damage and restores ATR signaling in CCND1-depleted MCL cells. We showed that depletion of CCND1 in MCL cell lines resulted in DNA double-strand breaks, reduced cell survival and increased sensitivity to DNA damaging agents (Mohanty *et al.*, 2016, see appendix). CCND1 KD also increased mRNA and protein levels of C53, a known negative regulator of CHEK1. Remarkably, depletion of C53 rescued growth inhibition and apoptosis in CCND1-
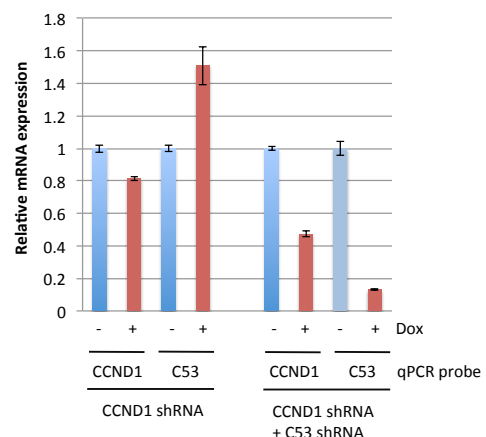
silenced cells, suggesting a potential role for C53 in mediating cytotoxicity of CCND1 KD. To further investigate the role of C53, we asked whether C53 depletion also rescue other phenotypes associated with CCND1 KD such as DNA damage and defective ATR-CHEK1 signaling. Using the inducible CCND1 KD and CCND1/C53 double-KD (DKD) UPN-1 lines previously generated (Fig. 1), we found that C53 depletion reduced gamma-H2AX levels in CCND1 KD cells after hydroxyurea (HU) exposure (Fig. 2). Consistent with reduced gamma-H2AX levels, phosphorylation of the upstream kinases ATM and CHEK2 were also reduced in CCND1/C53 DKD cells under the same HU exposure condition (Fig. 2).
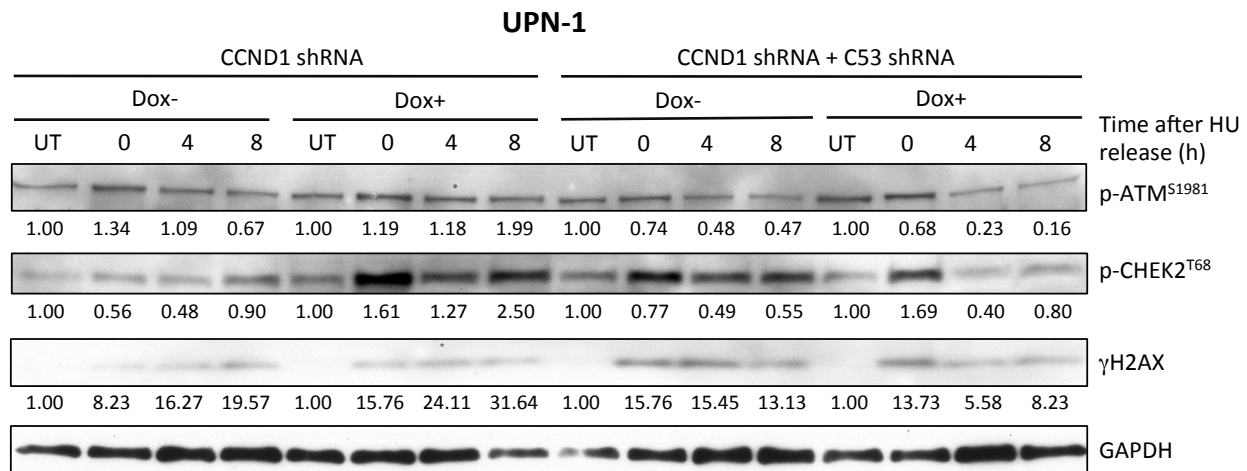
UPN-1

	CCND1 shRNA								CCND1 shRNA + C53 shRNA								
	Dox-				Dox+				Dox-				Dox+				
	UT	0	4	8	UT	0	4	8	UT	0	4	8	UT	0	4	8	Time after HU release (h)
p-ATM^{S1981}	1.00	1.34	1.09	0.67	1.00	1.19	1.18	1.99	1.00	0.74	0.48	0.47	1.00	0.68	0.23	0.16	
p-CHEK2^{T68}	1.00	0.56	0.48	0.90	1.00	1.61	1.27	2.50	1.00	0.77	0.49	0.55	1.00	1.69	0.40	0.80	
γH2AX	1.00	8.23	16.27	19.57	1.00	15.76	24.11	31.64	1.00	15.76	15.45	13.13	1.00	13.73	5.58	8.23	

Figure 2. C53 depletion reduces DNA damage in CCND1 KD cells. UPN-1 cells transduced with CCND1 shRNA or both CCND1 shRNA and C53 shRNA were induced with doxycycline (Dox) for 3 days to induce shRNA expression. Cell were then treated with 1mM of hydroxyurea (HU) for 16 h followed by washing and incubation in normal growth medium. Cell lysates were prepared at indicated time after HU removal and immunoblotted with indicated antibodies. Numbers below bands are relative densitometric values after normalization to GAPDH and untreated (UT) controls.

We next determined whether depletion of C53 would restore defects in ATR and CHEK1 signaling in CCND1-silenced cells. CCND1 KD and CCND1/C53 DKD UPN-1 cells were treated with etoposide overnight and ATR phosphorylation was assessed at differeent time points by immunoblot analysis. Under this condition, ATR phosphorylation in CCND1 KD cells was increased at 4 h but then reduced by 8 h after etoposide removal (Fig. 3). In contrast, ATR phosphorylation remained high in CCND1/C53 DKD cells at 8 h after etoposide removal (Fig. 3). These data indicate that depletion of C53 restore ATR activity in CCND1 KD cells after etoposide exposure.
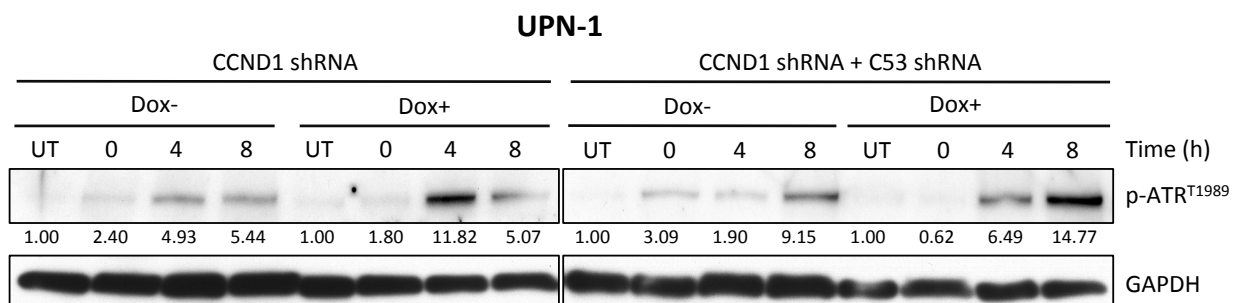
UPN-1

	CCND1 shRNA								CCND1 shRNA + C53 shRNA								
	Dox-				Dox+				Dox-				Dox+				
	UT	0	4	8	UT	0	4	8	UT	0	4	8	UT	0	4	8	Time (h)
p-ATR^{T1989}	1.00	2.40	4.93	5.44	1.00	1.80	11.82	5.07	1.00	3.09	1.90	9.15	1.00	0.62	6.49	14.77	

Figure 3. C53 depletion restores ATR activity in CCND1 KD cells. CCND1 KD and CCND1/C53 DKD UPN-1 cells as described in Figure 2 were treated with 500 nM of etoposide for 16 h followed by washing and incubation in normal growth medium. Cell lysates were prepared at indicated time after etoposide removal and immunoblotted with indicated antibodies. Numbers below bands are relative densitometric values after normalization to GAPDH and untreated (UT) controls.
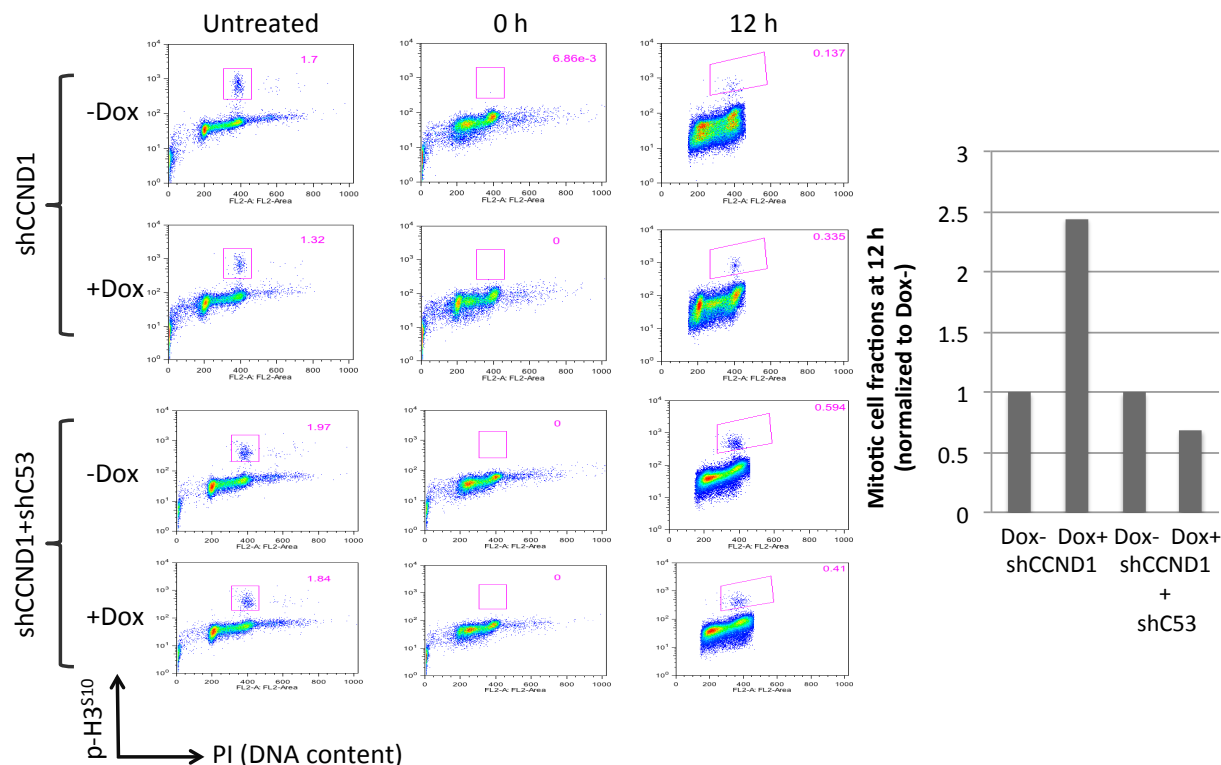
Figure 4. C53 depletion prevents premature mitotic entry in CCND1 KD cells. CCND1 KD and CCND1/C53 DKD UPN-1 cells as described in Figure 2 were treated with 500 nM of etoposide for 16 h followed by washing and incubation in normal growth medium. Cells at indicated time after etoposide removal were stained with p-histone H3 Ser10 antibody (detects mitotic cells) and propidium iodide (detects DNA content) and analyzed by flow cytometry. Gates drawn on p-H3 positive cells indicate mitotic cells. Bar graph on the right shows mitotic cell fractions after normalization to Dox- samples.

Defective ATR and CHEK1 signaling in CCND1 KD cells may lead to premature mitotic entry. To confirm this prediction and to assess the effect of C53 KD on this phenotype, we treated CCND1 KD and CCND1/C53 DKD cells with etoposide overnight followed by washing and incubation in normal growth medium. Compared to untreated cells, etoposide-treated cells were arrested at the G2 phase up to 12 h after drug removal. However, in CCND1 KD cells, there was 2.5-fold increase in mitosis, as detected by phosphorylation of Ser10 in histone H3 (Fig. 4). However, increased mitotic entry in CCND1 KD cells was abrogated when C53 was depleted (Fig. 4). This finding further supports a role for C53 in regulating ATR signaling as shown in Figure 3.

C53 overexpression did not phenocopy CCND1 KD. In preliminary studies, we showed that depletion of CCND1 in MCL cells led to increased C53 expression, defective CHEK1 signaling and increased apoptosis. Apoptotic cell death in CCND1 KD cells was rescued by depletion of C53. We also found that overexpression of specific C53 isoforms E or B in HEK-293T cells deregulated CHEK1 or CHEK2 signaling, respectively, following induced DNA damage. To further evaluate the role of C53 in MCL cells, we conditionally expressed the FLAG-tagged C53 isoform B or E in UPN-1 cells and determined the effects of C53 overexpression on cell growth and CHEK1 signaling. In contrast to our prediction, overexpression of either isoform B or E increased cell proliferation (Fig. 5A, B) and had little effect on sensitivity of UPN-1 cells to DNA damaging agents (Fig. 6). Further analysis revealed that neither forced expression of isoform E or D led to defective CHEK1 phosphorylation after recovering from HU exposure (Fig. 7A) or under continuous HU exposure, respectively (Fig. 7B). These data indicate that overexpression of C53 alone is not sufficient to phenocopy CCND1 KD.
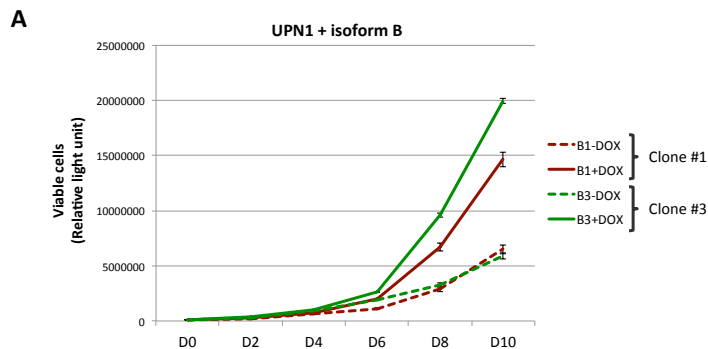
4

A

UPN1 + isoform B

B

UPN1 + isoform E

Figure 5. C53 overexpression promotes cell proliferation. UPN-1 cells were transduced with dox-inducible C53 isoform B or E and selected with puromycin. After Dox induction, cell growth was assessed at indicated time (in days) using the Celltiter Glo assay (Promega). Shown are means of 3 different experiments for 2 independent clones of isoform B (in A) and 3 clones of isoform E (in B). Error bars, SD.
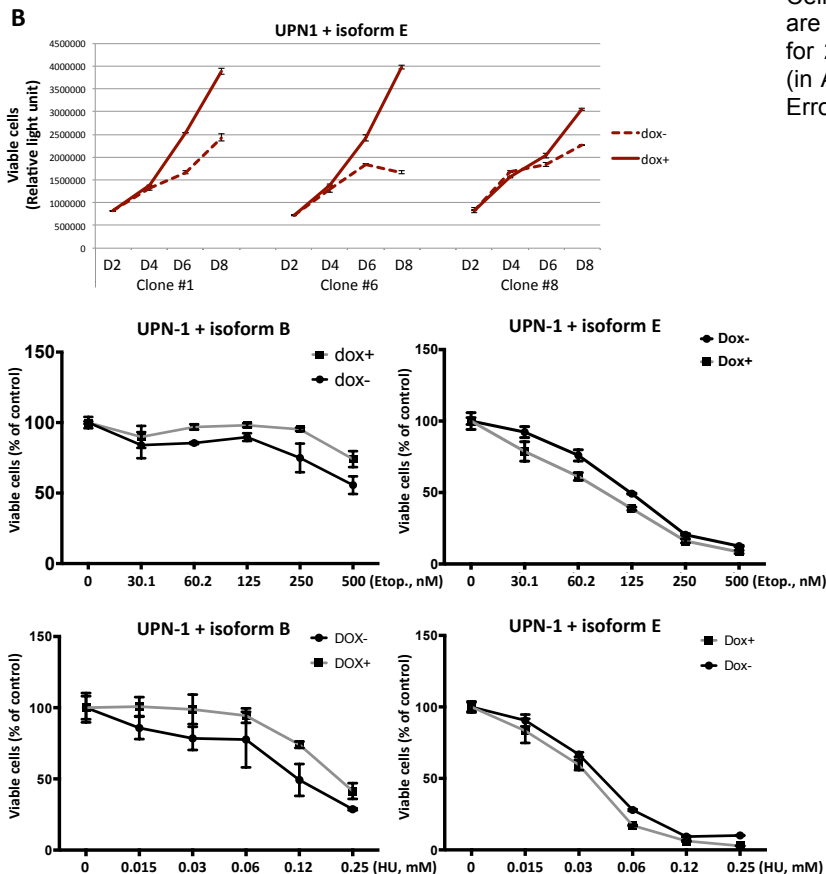

UPN-1 + isoform B

UPN-1 + isoform E

Figure 6. Effects of C53 overexpression on sensitivity to chemotherapeutic agents. UPN-1 cells expressing Dox-inducible C53 isoform B or E were treated with increasing doses of etoposide (Etop) or hydroxyurea (HU) for 72 h. Cell viability of control or Dox-induced cells was analyzed by the Celltiter Glo assay. Shown are means of 3 independent experiments. Error bars, SD.
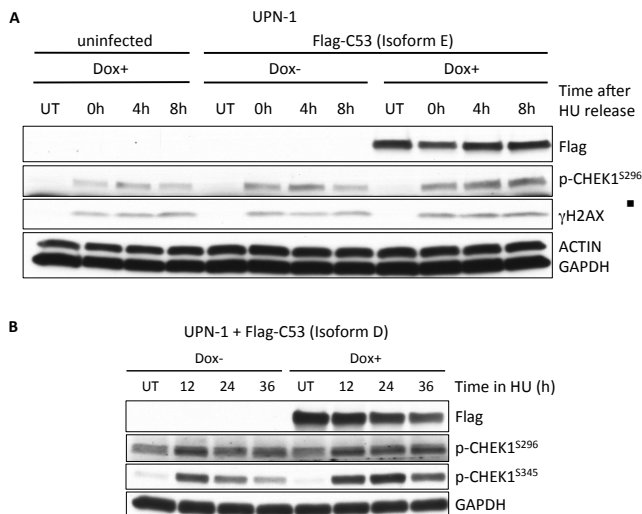

Figure 7. Effects of C53 overexpression on CHEK1 phosphorylation. UPN-1 cells expressing Dox-inducible C53 isoform E or D were induced with Dox for 3 days to induce C53 expression. Cells were then treated with 1mM of hydroxyurea (HU) for 16 h followed by washing and incubation in normal growth medium (A) or remained in HU for indicated times (B). Cell lysates were prepared at indicated time after HU removal and immunoblotted with indicated antibodies.

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In summary, we have shown that C53 depletion reduces DNA damage and restores ATR signaling in CCND1-depleted MCL cells. These results are consistent with the rescue of CCND1/C53 DKD cells from apoptosis and suggest a role for C53 in mediating cytotoxicity of CCND1 KD. However, overexpression of C53 isoforms in the MCL cell line UPN-1 did not recapitulate CCND1 KD phenotypes such as increased apoptosis, DNA damage and sensitivity to DNA damaging agents. It is possible that C53 overexpression alone may not be sufficient to phenocopy CCND1 KD, which may also affect other pro-survival factors in addition to upregulation of C53. Alternatively, C53 may mediate cytotoxicity of CCND1 KD not at the protein, but RNA levels. In support of this possibility, we observed that C53 shRNA efficiently reduced C53 mRNA but had little effect on the protein levels (Fig. 1 and data not shown). In addition, our unpublished data indicate that C53 is a very stable protein as assessed by cyclohexamide-based stability assay. We will address these possibilities in the plan outlined below.

What opportunities for training and professional development has the project provided?
The PI, Vu Ngo, has attended the following workshops and conferences to present research finding related to the project:

Gabrielle's Angel Foundation for Cancer Research Annual Symposium. 2015 Miami, Florida. Presentation title: The role of cyclin D1 in genomic stability and therapeutic implications in mantle cell lymphoma.
Lymphoma Research Foundation Workshop on Mantle Cell Lymphoma. 2016 Atlanta, Georgia. Presentation title: The role of cyclin D1 mutations in mantle cell lymphoma.
International Lymphoma Study Group (ILSG). 2016 Arcadia, California. Presentation title: Cyclin D1 mutations and ibrutinib resistance in mantle cell lymphoma.
ASH Meeting on Lymphoma Biology 2016, Colorado Springs, Colorado. Presentation title: Mechanisms of Ibrutinib Insensitivity in Mantle Cell Lymphoma.

How were the results disseminated to communities of interest?
Nothing to Report.

What do you plan to do during the next reporting period to accomplish the goals?
Our plan for the next reporting period includes a change in Specific Aim 1c (Task 1c in SOW) and the original Specific Aim 2a (Task 2a in SOW). Specifically, we will:

Determine whether C53 overexpression phenocopies CCND1 KD using alternative approaches (months 13-18).
Since overexpression of isoform B or E does not induce apoptosis or increase sensitivity to cytotoxic agents, we will repeat these experiments in the context of CCND1 knockdown. We expect that overexpression of C53 isoforms would enhance cytotoxicity of CCND1 KD and increase sensitivity of CCND1 KD cells to DNA damaging agents. We will also determine whether overexpressed C53 isoforms further disrupt ATR and CHEK1 signaling in CCND1 KD cells.

As an alternative approach, we will determine whether noncoding RNA (ncRNA) species generated at the *CDK5RAP3* gene locus may play a role in mediating the CCND1 KD phenotypes. We searched publicly available database of human noncoding RNAs and found evidence of ncRNA expression at the *CDK5RAP3* genomic region (http://lisanwanglab.org/DASHR/smdb.php#tabHome). These ncRNAs range from 20 to over 250 ribonucleotides long and reside at 5' UTR, exons, introns and 3' UTR of *CDK5RAP3*. Although expressed at low levels, their expression are detectable in normal B cells and in B-cell rich spleen tissue. To determine whether these ncRNA are present or induced by CCND1 KD in MCL cells, we will perform next generation sequencing for total RNA (RNA-seq) in control and CCND1 KD UPN-1 cells. If these potential ncRNAs are induced by CCND1 depletion, we will clone and express them in MCL cell lines (UPN-1 and JEKO-1) and determine if they produce phenotypes similar to CCND1 KD, including DNA damage, defective ATR-CHEK1 signaling and

sensitization to cytotoxic agents.

Determine whether CCND1 regulates C53 transcription (months 18-24).
- Generate UPN-1 cell lines that express either Flag-tagged CCND1 or empty vector control and perform chromatin immunoprecipitation (ChIP) with anti-Flag antibody.
- Use quantitative PCR with *C53 promoter*-specific primers to detect the *C53* promoter DNA in immunoprecipitates generated above.
- Assess *C53* transcriptional activity using a C53 promoter-luciferase reporter system.
- Determine whether CCND1 over-expression suppresses the reporter activity by co-expressing the dual-luciferase reporter constructs (both C53 promoter-firefly luciferase and control-Renilla luciferase) with increasing amount of CCND1-expressing vector in HEK-293 cells.

4 IMPACT:
What was the impact on the development of the principal discipline(s) of the project?
Nothing to Report.
What was the impact on other disciplines?
Nothing to Report.
What was the impact on technology transfer?
Nothing to Report.
What was the impact on society beyond science and technology?
Nothing to Report.

5 CHANGES/PROBLEMS:

Changes in approach and reasons for change
We will replace experiments in the original Specific Aim 1c (Task 1c in SOW) with new experiments to "*determine whether C53 overexpression phenocopies CCND1 KD using alternative approaches*," as described above. This change is necessary because we need to know why C53 depletion could rescue cytotoxicity of CCND1 KD but C53 overexpression did not phenocopy CCND1 KD. Answers to this question would clarify the role of C53 in mediating CCND1 KD phenotypes. We choose to replace experiments in the original Specific Aim 1c because these experiments are mainly to confirm in MCL cells the effects of C53 on CHEK1 and CHEK2 activities, which have been previously described in HeLa cells by the Li's group (Jiang et al. *Cell Res.* 2009 19:458).

Actual or anticipated problems or delays and actions or plans to resolve them: N/A

Changes that had a significant impact on expenditures: N/A

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents: N/A

6 PRODUCTS:
Publications, conference papers, and presentations
> **Journal publications.**
> Mohanty S, Mohanty A, Sandoval N, Tran T, Bedell V, Wu J, Scuto A, Weisenburger D, Ngo VN. Cyclin D1 depletion induces DNA damage in mantle cell lymphoma lines. *Leuk Lymphoma.* 2016 Jun 24:1-13 [Epub ahead of print]. *Acknowledgement of federal support: yes.*

> **Conference papers**
> 2016 The role of cyclin D1 mutations in mantle cell lymphoma. *Lymphoma Research Foundation Workshop on Mantle Cell Lymphoma.* Atlanta, Georgia.

> **Presentations**

Mechanisms of Ibrutinib Insensitivity in Mantle Cell Lymphoma. ASH Meeting on Lymphoma Biology 2016, Colorado Springs, CO.

Cyclin D1 mutations and ibrutinib resistance in mantle cell lymphoma. *International Lymphoma Study Group (ILSG)* 2016. Arcadia, California.

Website(s) or other Internet site(s)
N/A
Technologies or techniques
N/A
Inventions, patent applications, and/or licenses
N/A
Other Products
N/A

7 PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS
What individuals have worked on the project?

Name:	*Vu Ngo*
Project Role:	*PI*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*4*
Contribution to Project:	*No change*

Name:	*Atish Mohanty*
Project Role:	*Postdoctoral Fellow*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*12*
Contribution to Project:	*No change*

Name:	*Natalie Sandoval*
Project Role:	*Research Associate*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*3*
Contribution to Project:	*No change*

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
Nothing to Report.
What other organizations were involved as partners?
Nothing to Report.

8 SPECIAL REPORTING REQUIREMENTS
COLLABORATIVE AWARDS: *N/A*
QUAD CHARTS: *N/A*

APPENDICES:
Reprints of manuscripts

ORIGINAL ARTICLE: RESEARCH

Cyclin D1 depletion induces DNA damage in mantle cell lymphoma lines

Suchismita Mohanty[a]*, Atish Mohanty[a]*, Natalie Sandoval[a], Thai Tran[b], Victoria Bedell[c], Jun Wu[d], Anna Scuto[c], Joyce Murata-Collins[c], Dennis D. Weisenburger[c] and Vu N. Ngo[a]

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ABSTRACT

Elevated cyclin D1 (CCND1) expression levels in mantle cell lymphoma (MCL) are associated with aggressive clinical manifestations related to chemoresistance, but little is known about how this important proto-oncogene contributes to the resistance of MCL. Here, we showed that RNA interference-mediated depletion of CCND1 increased caspase-3 activities and induced apoptosis in the human MCL lines UPN-1 and JEKO-1. *In vitro* and xenotransplant studies revealed that the toxic effect of CCND1 depletion in MCL cells was likely due to increase in histone H2AX phosphorylation, a DNA damage marker. DNA fiber analysis suggested deregulated replication initiation after CCND1 depletion as a potential cause of DNA damage. Finally, in contrast to depletion or inhibition of cyclin-dependent kinase 4, CCND1 depletion increased chemosensitivity of MCL cells to replication inhibitors hydroxyurea and cytarabine. Our findings have an important implication for CCND1 as a potential therapeutic target in MCL patients who are refractory to standard chemotherapy.

Introduction

Mantle cell lymphoma (MCL) is an incurable B-cell malignancy that accounts for 6–8% of all non-Hodgkin lymphomas. Most MCL patients respond initially to conventional chemotherapy, but then quickly develop resistance, leading to a median survival of 3–5 years.[1,2] MCL is characterized by the recurrent t(11;14) chromosomal translocation,[3] resulting in CCND1 overexpression. CCND1 binds and activates cyclin-dependent kinase (CDK) 4 or CDK6 to mediate phosphorylation-dependent degradation of the major G1 checkpoint protein retinoblastoma protein (RB) and promote S-phase entry.[4–6] Other D-type cyclins, cyclin D2 (CCND2) and cyclin D3 (CCND3), which also associate with CDK4 and CDK6,[7] are less common in MCL, and their expression are more associated with CCND1 negative cases.[8,9] Salaverria et al.[9] has reported 55% of MCL cases that lacked CCND1 expression had chromosomal rearrangement at the CCND2 locus. Translocation at the CCND3 locus has been reported only in one CCND1 negative case [8] and this translocation appear more common in other B-cell lymphomas.[10] While the CDK4/RB-dependent function of

CCND1 is important for MCL development, targeting this pathway by inactivating CDK4/6 kinase causes G1 arrest with little cytotoxicity in the lymphoma cells.[11–15]

Overexpression of CCND1 has been shown to correlate with increased chemoresistance and poor survival in many cancers.[16–19] In MCL, Rosenwald et al.[20] demonstrated that elevated CCND1 expression levels are associated with high tumor cell proliferation and aggressive clinical manifestations, likely related to chemoresistance. In another study by Tiemann et al.,[21] the authors showed that depletion of CCND1 using siRNA increased the sensitivity of MCL cell lines to etoposide or doxorubicin. These reports suggest an important role of CCND1 in protecting MCL cells against conventional chemotherapeutic agents, but the mechanisms underlying this function are largely unknown. In this study, we address this question by hypothesizing that CCND1 is essential for genomic stability in MCL tumors. We tested the hypothesis by determining the effects of CCND1 depletion on DNA stability and cellular response to the DNA damaging agents hydroxyurea (HU) or cytarabine in MCL lines

and primary MCL cells *in vitro* and in a xenotransplant model.

Materials and methods

Cell lines and culture conditions

Human MCL lines UPN-1 and JEKO-1 were kindly provided by Dr Louis Staudt. The UPN-1 cell line was confirmed to carry the cyclinD1/IGH fusion gene or t(11;14) translocation and other chromosomal rearrangements, as previously described,[22] by metaphase fluorescence *in situ* hybridization (FISH) analysis (Supplementary Figure S1). JEKO-1 cells were previously reported to carry the t(11;14) translocation.[23] Cells were cultured in RPMI-1640 medium (Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum and penicillin/streptomycin and maintained in a humidified, 5% CO_2 incubator at 37 °C.

Primary MCL preparation

Viably cryo-preserved MCL cells were obtained from the tumor bank of the Pathology Department of City of Hope as de-identified samples after approval by the Institutional Review Board. Cells were briefly thawed in 37 °C water bath, washed in RPMI-1640 medium and subsequently cultured in RPMI-1640 medium supplemented with 20% fetal bovine serum.

Antibodies and chemicals

The following antibodies were used: pRB-S807/811 (Cell Signaling Technology, Danvers, MA), pRBL2-S672, RBL2 (Abgent, Inc., San Diego, CA), CCND1, CDK4, CDK6, GAPDH, β-ACTIN (Santa Cruz, Dallas, TX), RB (BD Biosciences, San Jose, CA), and γH2AX (Millipore, Billerica, MA). The following chemicals were used: PD-0332991 (Selleck Chemicals, Houston, TX), hydroxyurea, cytarabine, and aphidicolin (Sigma, St. Louis, MO).

RNA interference reagents and siRNA transfection

All shRNA constructs and sequences were obtained from a previously generated shRNA library [24] and listed in Supplementary Table S1. SMARTpool ON-TARGETplus siRNA targeting CCND1 or CDK4 was purchased together with scramble control siRNA from Dharmacon (Dharmacon, Lafayette, CO). Two rounds of siRNA transfection were performed on days 0 and 2 as recommended by the manufacturer.

Western blotting

Cells were lysed in the presence of protease inhibitors (Roche, Basel, Switzerland) and phosphatase inhibitors (Sigma, St. Louis, MO) for 30 min. Lysates were cleared by centrifugation and protein concentrations were determined by BCA protein assay (Life Technologies, Grand Island, NY). About 30–80 μg of lysates were separated by 4–15% SDS–PAGE and immobilized on the nitrocellulose membranes for immunoblotting.

Viability and apoptosis measurements

Cell viability was performed either by using the TC20 Automated Cell Counter (Biorad, Hercules, CA), based on the trypan blue exclusion method, or by a Celltiter-Glo assay (Promega, Madison, WI) following instructions by the manufacturer. To measure apoptosis, cells were stained with anti-active Caspase-3 and anti-cleaved PARP antibodies (BD Biosciences, San Jose, CA) or analyzed by Annexin V-based methods using Annexin V Apoptosis Detection Kit (eBioscience, Inc., San Diego, CA).

Retroviral transduction

A retroviral vector and a mixture of helper plasmids for viral envelope and *gag/pol* were transfected into HEK293T cells using Lipofectamine2000 (Life Technologies, Grand Island, NY). Retroviral supernatants were harvested 48 h after transfection and were used to transduce ectropic receptor-expressing target cells by centrifugation at 1360*g* for 1 h in 4 μg/ml polybrene (Sigma, St. Louis, MO).

Rescue of shRNA toxicity by cDNA complementation

Complementation studies were performed in the UPN-1 cell line. Briefly, cells were transduced with retroviral vectors that express a 3′-UTR-directed CCND1 shRNA. CCND1 shRNA-transduced cells were subsequently infected with retroviruses co-expressing a wild-type CCND1 cDNA and hygromycin resistant gene. Hygromycin-selected cells were induced with doxycycline to induce shRNA expression and apoptosis was monitored over time by active caspase-3/cleaved PARP staining.

Immunofluorescence

Cells were fixed with 2% paraformaldehyde and permeabilized with 0.5% Triton X-100, followed by

blocking with 2% BSA and 0.05% Tween-20 in phosphate buffered saline. Cells were stained with primary antibodies and then with fluorescently labeled secondary antibodies, followed by nuclear staining with 4′,6-diamidino-2-phenylindole (DAPI) (Life Technologies, Grand Island, NY).

Xenograft study

Mouse care and experimental procedures were performed in accordance with established institutional guidelines and approved protocols from the Institutional Animal Care and Use Committee of the City of Hope. Five millions of CCND1 shRNA-transduced UPN-1 cells were subcutaneously injected into dorsal skin area of 7- to 8-week-old immunodeficient NOD/SCID/IL2R-Gamma (NSG) mice (Jackson Laboratories, Bar Harbor, ME). After tumors reached average size of ∼5 mm, sucrose or doxycycline (Sigma, St. Louis, MO) was added to mouse drinking water and tumor growth was monitored every other day by caliper measurements.

DNA combing assay

Exponentially growing cells were pulse-labeled with 50 μM iododeoxyuridine (IdU) followed by 100 μM chlorodeoxyuridine (CldU) for 20 and 40 min each. Labeled cells were harvested and DNA fiber spreads were processed as described.[25]

Cell cycle analysis

Cells were cultured with bromodeoxyuridine (BrdU) for 30 min, washed in PBS, and fixed overnight in 70% ethanol. Labeled cells were permeabilized in Perm/Wash buffer (BD-Biosciences) and treated with 4N hydrochloric acid and 1% Triton X-100. Acid was neutralized by sodium tetraborate (pH 8.5). Cells were incubated with an anti-BrdU antibody (BD-Biosciences) followed by washing and resuspending in RNAse/PI-staining solution (Life Technologies). Flow cytometric analysis was performed on a FACSCalibur (BD-Biosciences).

Statistical analyses

A two-tailed Student's t-test was used for normally distributed values. For non-normally distributed values, a two-tailed Mann–Whitney test was used. A chi-squared test or a two-sided Fisher's exact test was used for categorical data. All tests were analyzed using Prism Version 6.0b (GraphPad Software, Inc.). $p < 0.05$ was considered significant. Complete methods are available in Supplementary Methods.

Results

CCND1 is required for the survival of MCL cells

To examine CCND1 function in the proliferation and survival of MCL, we depleted this protein in the human MCL lines UPN-1 and JEKO-1 using short-interfering (si)RNA. To distinguish other roles of CCND1 from those of its well-characterized pathway through CDK4 or CDK6, we also depleted CDK4 in parallel experiments, as the cell lines we chose do not express CDK6 (Figure 1A). Protein knockdown were confirmed by immunoblot analysis (Figure 1B). Depletion of CCND1 or CDK4 after 7 days decreased cell proliferation in both cell lines (Figure 1C). To determine whether the growth inhibition observed in these cells was due to cytotoxic effects of gene silencing, the apoptotic marker active caspase 3 was evaluated by flow cytometry. Compared to controls, CCND1 depletion induced apoptosis in UPN-1 and JEKO-1 cells more than CDK4 depletion (Figure 1D and E). As an alternative method to evaluate cell death, Annexin V and propidium iodide (PI) positive cells were analyzed by flow cytometry on day 5 after siRNA transfection. Consistent with the results shown in Figure 1D and E, depletion of CCND1 but not CDK4 significantly increased Annexin V and PI staining in UPN-1 and JEKO-1 cells (see DMSO-treated samples in Figures 5A and B).

To determine that little toxicity in UPN-1 and JEKO-1 cells was not due to partial CDK4 knockdown, cells were treated with the CDK4/6-specific inhibitor PD-0332991.[13] The effectiveness of this drug was verified by its ability to block the phosphorylation on RB, a known target of CDK4/6. Since UPN-1 cells do not express RB,[26] the alternative CDK4/6 target protein RB-like 2 (RBL2) was evaluated. In a dose-dependent manner, PD-0332991 effectively blocked the phosphorylation of RB or RBL2, leading to decreased cell proliferation in both cell lines (Figure 1F and G). However, compared to DMSO-treated controls, PD-0332991 only modestly induced apoptosis in UPN-1 and JEKO-1 cells (Figure 1H and I). These results were similar to the effects of siRNA-mediated CDK4 depletion shown in Figure 1D and E.

We next used shRNA as an alternative gene knockdown method to confirm the effects of CCND1 depletion. UPN-1 and JEKO-1 cells were transduced with retroviral vectors that co-expressed control or CCND1 shRNA and enhanced green fluorescence protein (eGFP). The proliferation and/or survival of transduced
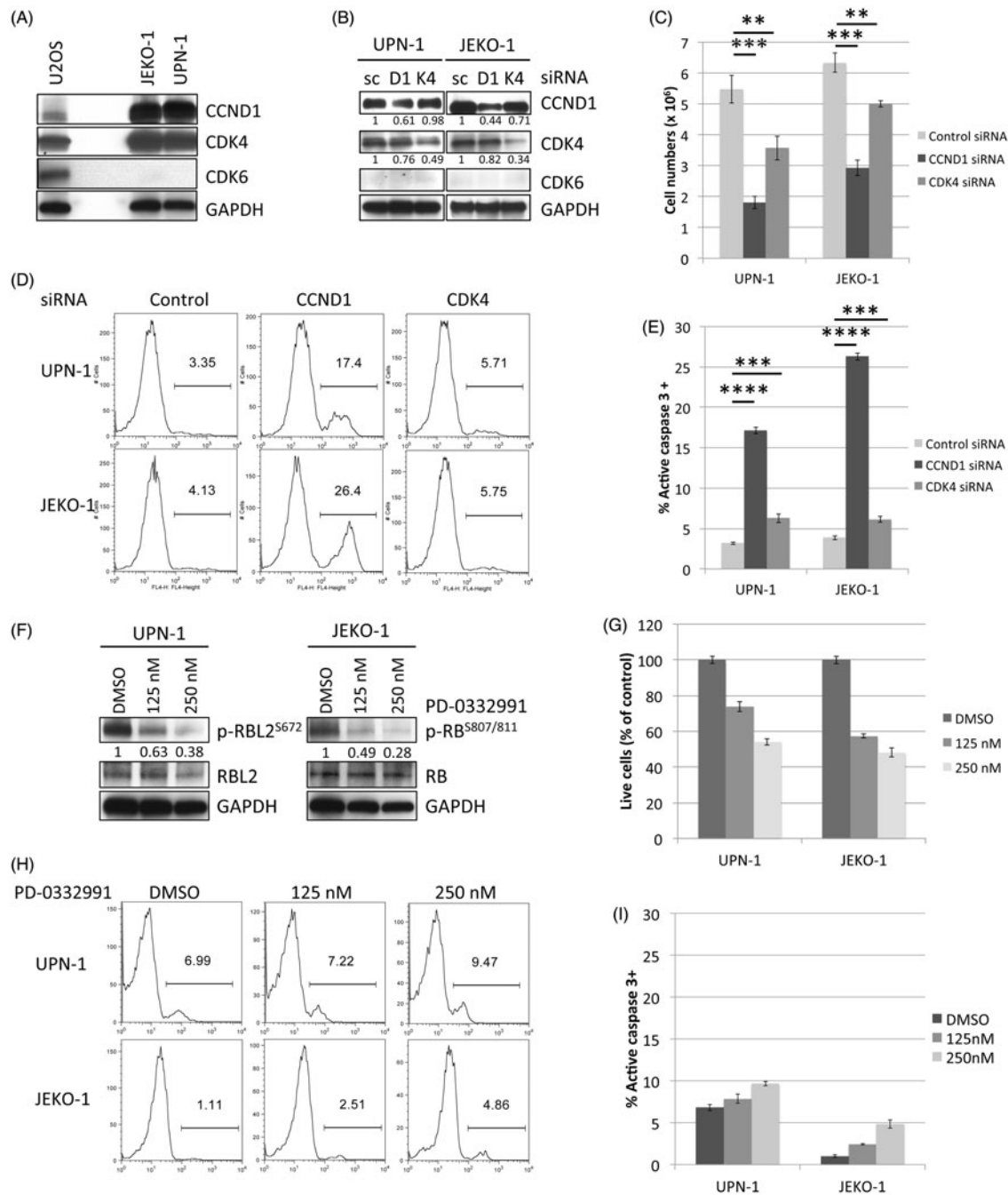
cells were then determined by monitoring the fractions of eGFP positive cells over time using flow cytometry. Compared to controls, CCND1 shRNA significantly reduced the viability of both UPN-1 and JEKO-1 cells in a time-dependent fashion (Figure 1J). Because CCND1 depletion is toxic in the MCL lines used in our study, we generated a stable UPN-1 cell line that conditionally expresses CCND1 shRNA, using a doxycycline (Dox)-inducible retroviral expression system.[24] To validate that the toxicity of the CCND1 shRNA was not due to an off-target effect, we demonstrated that a cDNA vector encoding wild-type CCND1 was able to

rescue apoptosis induced by a 3′-UTR-directed CCND1 shRNA (Figure 1K). We next compared the effects of CCND1 and CDK4 shRNA-mediated knockdown on proliferation and survival using stable UPN-1 cell lines that conditionally express CCND1 or CDK4 shRNA. To ensure similar CCND1 and CDK4 knockdown efficiencies, cells with similar expression levels of the co-expressed eGFP marker were sorted by flow cytometry to normalize shRNA expression (Supplementary Figure S2). As expected, shRNA-mediated depletion of CCND1 in UPN-1 cells led to more growth inhibition and apoptosis than depletion of CDK4 in multiple time points
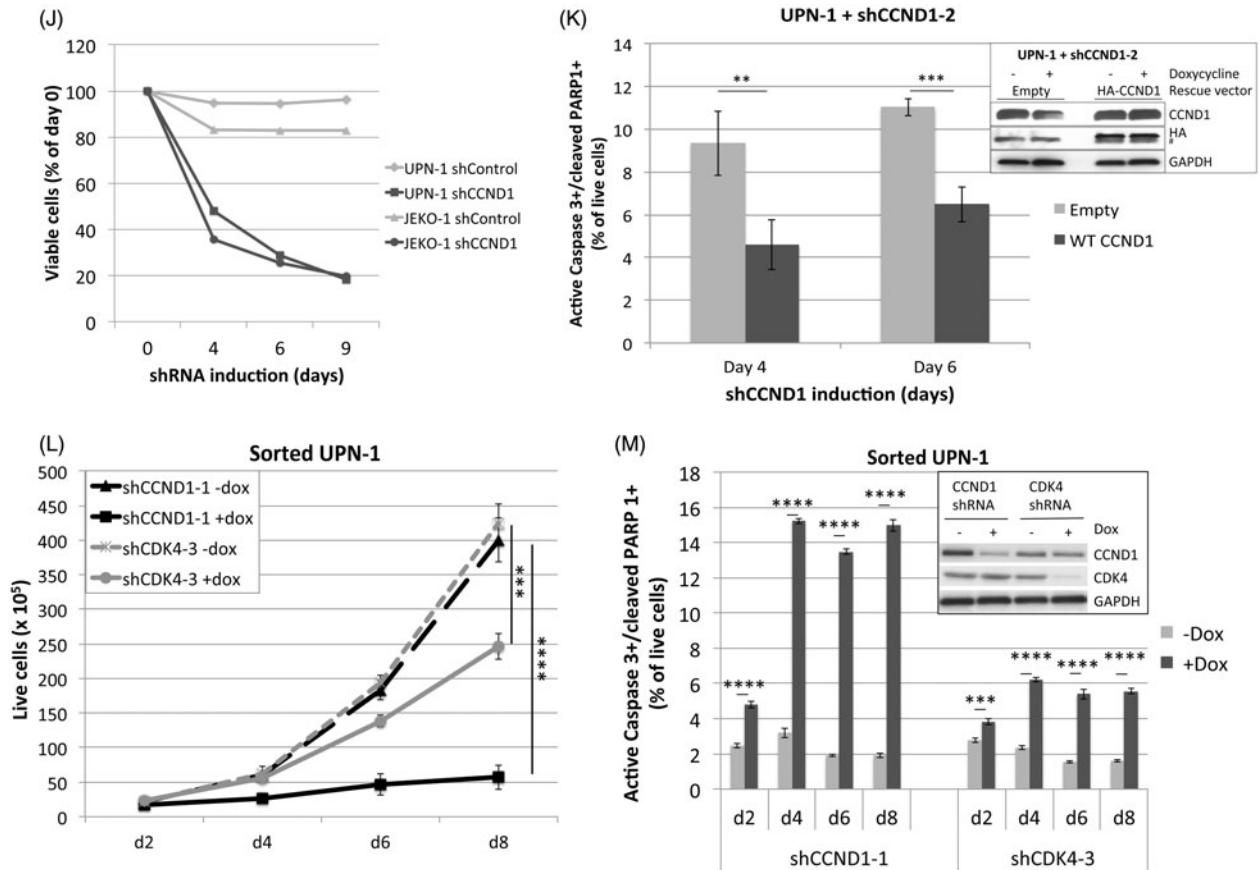
Figure 1. CCND1 is required for survival of MCL cells. (A) Immunoblot shows UPN-1 and JEKO-1 cells express little CDK6 with U2-OS cells as a positive control for CDK6 expression. (B) Immunoblot shows protein knockdown in indicated MCL cell lines on day 4 after transfection with scramble control (sc), CCND1 (D1), or CDK4 (K4) siRNA. Numbers below the bands are relative densitometric values between target proteins and GAPDH loading control, followed by normalization to the control siRNA for each protein. (C) Effects of siRNA knockdown on cell proliferation. Shown are the numbers of viable cells as determined by trypan blue exclusion on day 7 after transfection with indicated siRNA. (D) Detection of apoptosis in indicated cell lines 7 days after transfection with indicated siRNA. Shown are representative histograms of live cells stained with the active caspase-3 antibody followed by fluorescence-activated cell sorting (FACS) analysis. (E) Quantification of active caspase 3-positive cell fractions described in (D). (F). Immunoblot analysis of indicated cell lines 48 h after treatment with indicated doses of PD-0332991. Inhibition of CDK4/6 activity by PD-0332991 was shown by dephosphorylation of the CDK4/6 target protein RB or RBL2. RB was absent in UPN-1 cells and, therefore, RBL2 was analyzed. Relative densitometric values were calculated as described in (B). (G) Effects of PD-0332991 on cell proliferation. Shown are percentages of viable cells compared to DMSO-treated cells as determined by the Celltiter-Glo assay 7 days after treatment with indicated doses of PD-0332991. (H) Detection of apoptosis in indicated cell lines after 7-day treatment with PD-0332991 as described in (D). (I) Quantification of active caspase 3-positive cell fractions described in (H). (J) Toxicity of CCND1 shRNA in MCL cell lines. Shown are the normalized fractions (to day 0 values) of GFP^{+}, shRNA-expressing cells relative to GFP^{-}, shRNA-negative fractions at indicated times. (K) Rescue of CCND1 shRNA toxicity by cDNA complementation. UPN-1 cells were transduced with a doxycycline (dox)-inducible 3′-UTR-directed CCND1 shRNA (shCCND1-2) and a rescue vector constitutively expressing HA-tagged, wild-type (WT) CCND1 coding regions or an empty vector. Apoptotic cells were assessed as described in (D) at indicated times after shRNA induction and shown as percentages of active caspase 3/cleaved PARP-1-positive cells. Inset, immunoblot verifies CCND1 overexpression or knockdown 2 days after shRNA induction. #Nonspecific bands. (L) Effects of CCND1 or CDK4 depletion on cell growth. Shown are the means of live cell numbers of control or shRNA-induced cells as determined by trypan blue exclusion on indicated days. (M) Apoptosis in UPN-1 cells transduced with indicated shRNA was detected at indicated times after shRNA induction by staining with active caspase-3 and cleaved PARP-1 antibodies. Inset, immunoblot confirms protein knockdown 2 days after shRNA induction. Values in line graphs or bar graphs are the means of three independent experiments. Error bars, SD. ****$p < 0.0001$; ***$p < 0.001$; **$p < 0.01$ by a two-tailed Student's t-test.
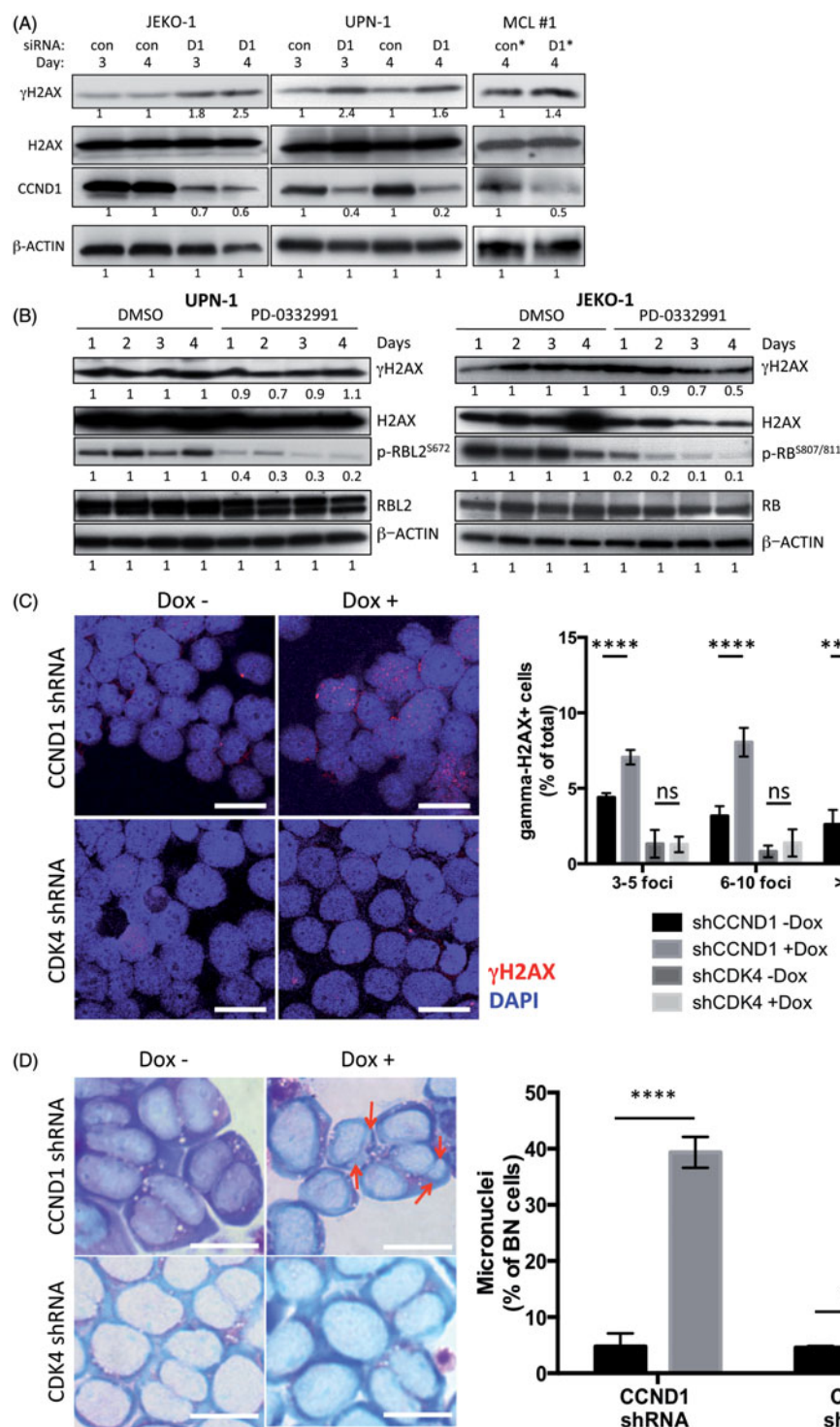
Figure 2. CCND1 is required for DNA stability in MCL cells. (A) CCND1 depletion induces γ-H2AX expression. Immunoblot analysis of indicated MCL cell lines on day three or four after transfection with control (con) or CCND1 (D1) siRNA. Primary MCL cells (MCL #1) with more than 95% of tumor content (see Supplementary Figure S4) were transduced with control or CCND1 shRNA-expressing lentivirus and analyzed by immunoblot on day 4 after transduction. Relative densitometric values were calculated as described in Figure 1B. (B) Effect of CDK4 inhibition on γ-H2AX expression. UPN-1 and JEKO-1 cells were treated with 250 nM of PD-0332991 for indicated times and analyzed by immunoblot. Relative densitometric values were calculated as described in Figure 1B. (C) CCND1 depletion induces DNA DSB foci. CCND1 or CDK4 shRNA-transduced UPN-1 cells were induced with Dox for four days. Shown are representative confocal immunofluorescence images of uninduced and induced cells stained with Alexa-Fluor 555-conjugated phospho-H2AX (S139) antibody (red) followed by nuclear staining with DAPI (blue). Scale bars, 20 μm. Bar graphs show the mean values of cell fractions that are positive for phospho-H2AX (γH2AX) foci from two independent experiments. Error bars, SEM. Foci of at least 1 μm in diameter were considered positive for γH2AX. On average, 500 cells from each group were counted. ****$p < 0.0001$ by a Chi-square test; ns, not significant. More γH2AX foci in uninduced CCND1 shRNA-transduced cells than in CDK4 shRNA-transduced cells indicate ''leakiness'' of the Dox inducible system in UPN-1 cells. (D) CCND1 depletion induces genomic instability. Representative bright-field images of uninduced or day-four induced UPN-1 cells treated with cytochalasin B followed by Giemsa nuclear staining. Scale bars, 20 μm. Bar graphs show the percentages of binucleated (BN) cells with micronucleus (MN) formation from two separate experiments. Error bars, SEM; ****$p < 0.0001$ by a two-sided Fisher's exact test. Approximately 200 binucleated (BN) cells from each group were counted.

starting on day 4 after Dox induction (Figure 1L and M). Altogether, we conclude that survival of UPN-1 and JEKO-1 cells is more dependent on CCND1 than on CDK4.

CCND1 is required for DNA stability in vitro and in a xenograft model of MCL

To determine whether apoptosis induced by CCND1 depletion was due to DNA damage, we evaluated DNA double-stranded break (DSB) formation by detecting histone H2AX phosphorylation on Ser139 (gamma-H2AX), an established DSB marker,[27] in UPN-1 and JEKO-1 cells transfected with CCND1 siRNA. Compared to controls, CCND1-depleted cells had increased gamma-H2AX expression on days 3 and 4 after siRNA (Figure 2A). In contrast, gamma-H2AX induction was not detected in these MCL lines treated with PD-0332991 (Figure 2B) or depleted of CDK4 (Supplementary Figure S3) for up to 4 days. Increased gamma-H2AX signals were also observed in primary MCL cells 4 days after transduction with CCND1 shRNA-expressing lentivirus (Figure 2A). The tumor content in this primary MCL sample was confirmed to have >95% of CD19 + CD5 + lymphoma cells (Supplementary Figure 4). As an alternative method of detecting DSBs, we used immunofluorescence to identify cells with nuclear gamma-H2AX foci. Using inducible knockdown UPN-1 cell lines generated above, we demonstrated that depletion of CCND1, but not CDK4, significantly increased the number of gamma-H2AX$^+$ foci (Figure 2C), which were more frequent in the S and G2 phases of the cell cycle (Supplementary Figure S5). These data indicate that CCND1, but not CDK4, plays an important role in preventing DNA damage in MCL cells.

DNA damage after CCND1 depletion in MCL cells prompted us to evaluate genomic stability in these cells. Using a cytokinesis-block micronucleus assay [28] to detect nuclear damage during mitotic segregation, we observed that depletion of CCND1, but not CDK4, significantly increased micronucleus formation in UPN-1 cells (Figure 2D). Further cytogenetic analysis of chromosomal aberrations by metaphase karyotyping following CCND1 silencing in UPN-1 cells revealed chromosomal breaks, chromatid breaks and recurrent chromosomal gaps or constrictions that are associated with fragile sites (Supplementary Figure S6, Supplementary Table S2).

We next evaluated the in vivo relevance of CCND1 inactivation by engrafting CCND1 shRNA-expressing UPN-1 cells into immunodeficient NOD/SCID/IL2R-Gamma (NSG) mice. CCND1 depletion resulted in reduced growth (Figure 3A–C) and increased apoptosis, as detected by active caspase 3 immunofluorescence (Figure 3E), in the transplanted tumors. Increased accumulation of gamma-H2AX foci was also observed in CCND1-depleted, but not control, xenografts (Figure 3D). These findings are consistent with the in vitro effects of CCND1 depletion in UPN-1 cells on cell growth, apoptosis and DNA stability described above. Together, these data indicate that CCND1 is essential for maintaining DNA stability in MCL cells and specifically in the UPN-1 cells both in in vitro and in vivo conditions.

CCND1 depletion leads to defective DNA replication in UPN-1 cells

In the absence of induced DNA damage, DNA breakage often occurs during replication stress, a process when DNA synthesis becomes inefficient.[29] To determine whether DNA damage in CCND1-depleted UPN-1 cells were related to defects in DNA replication, the DNA combing assay [25] was used to assess replication initiation. In this assay, CCND1- or CDK4-depleted UPN-1 cells were pulse-labeled with the thymidine substitutes iododeoxyuridine (IdU) and chlorodeoxyuridine (CldU) for 20 and 40 min each. DNA fibers were extracted from cell lysates and spread on glass slides for immunofluorescence analysis using antibodies specific for IdU or CldU. Compared to uninduced or CDK4-depleted cells, CCND1-depleted cells showed a markedly reduced distance between replication origins, indicating increased frequency of origin firing (Figure 4). Thus, CCND1 may have an important role in DNA replication initiation and reduced CCND1 expression levels may result in unscheduled origin firing and replication stress-related DNA breakage.

CCND1 depletion sensitizes MCL cells to DNA replication inhibitors

We next determined whether CCND1 plays a role in the chemoresistance of MCL cells to the replication inhibitors hydroxyurea (HU) or cytarabine. To do so, we depleted CCND1 by siRNA in UPN-1 and JEKO-1 cell lines for 3 days. Equal numbers of siRNA-transfected cells were re-plated and treated with increasing concentrations of HU or cytarabine for 2 days followed by evaluation of apoptosis using Annexin V-based flow cytometry. Knockdown of target proteins in these experiments was confirmed by immunoblot analysis (Figure 5A and B insets). CCND1 depletion increased sensitivity of UPN-1 and JEKO-1 cells to HU
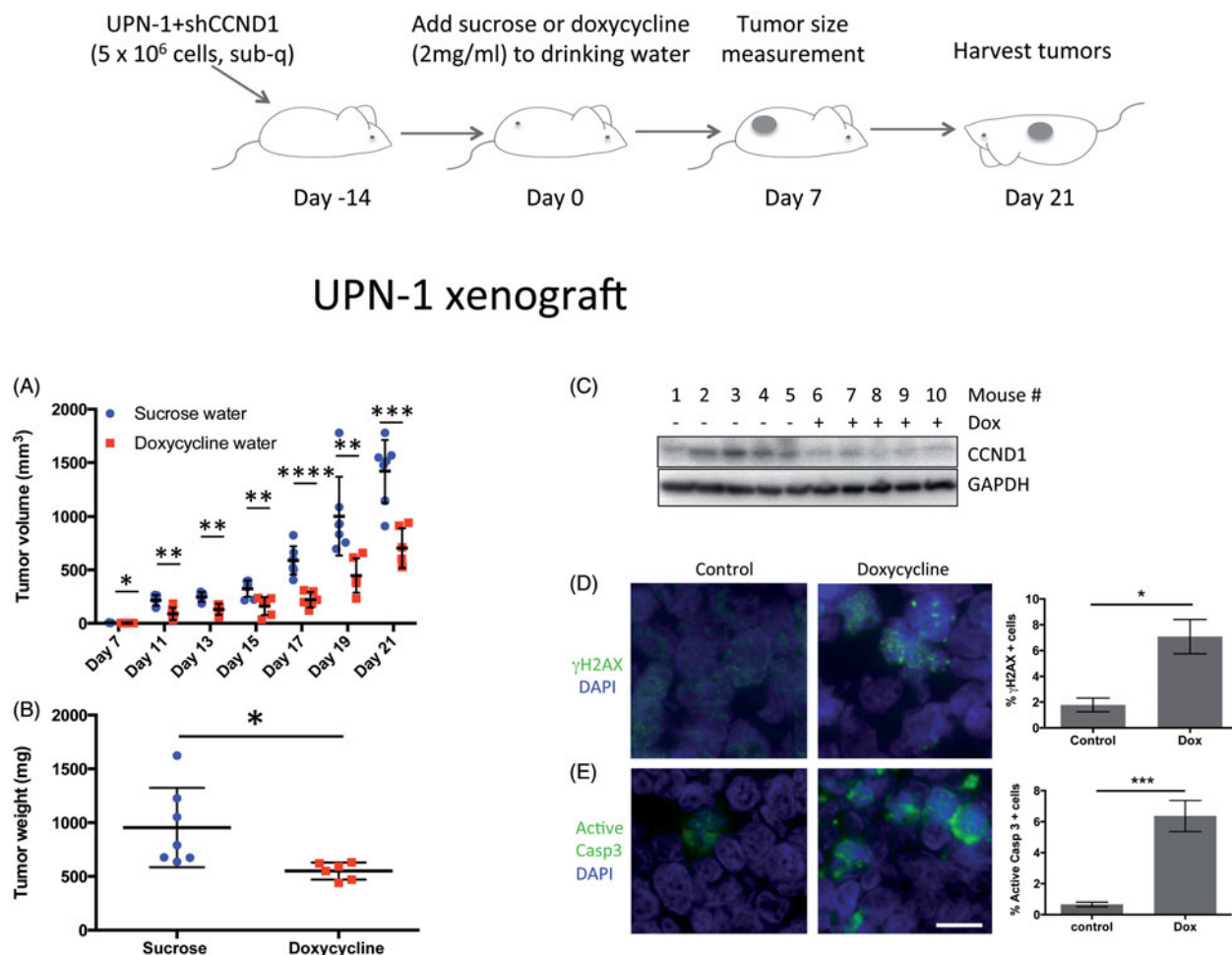
Figure 3. Effects of CCND1 depletion in a xenograft model of MCL. Five millions of CCND1 shRNA-transduced UPN-1 cells were injected subcutaneously into NSG mice and shRNA expression was induced by adding Dox to mouse drinking water. (A) Shown are the means of tumor volumes ($n = 7$). Error bars, SEM. (B) Shown are the means of tumor weights. Error bars, SD. (C) Lysates of MCL tumors harvested from control or Dox-induced mice on day 21 were immunoblotted with indicated antibodies. (D and E) Representative immunofluorescence images of tumor sections from control and Dox-induced groups were stained with indicated antibodies followed by fluorescently labeled secondary antibody and nuclear staining with DAPI. Scale bars, 10 μm. Bar graphs show the percentages of γH2AX+ or active caspase 3+ cells from control ($n = 3$) or Dox-induced ($n = 3$) xenografts. Foci with at least 1 μm in diameter were considered positive for γH2AX. At least 500 DAPI-positive cells were counted in non-overlapping fields of immunofluorescence images. Error bars, SD; ***$p < 0.001$; *$p < 0.05$ by a two-tailed Student's t-test.

(Figure 5A and B) or cytarabine (Figure 5C and D) dose-dependently. Using the inducible CCND1 knockdown UPN-1 cell line, we confirmed that induced CCND1 shRNA sensitized UPN-1 cells to HU or to another replication inhibitor aphidicolin (Figure 5E). Enhanced chemosensitivity of UPN-1 and JEKO-1 cells after CCND1 depletion was not due to CDK4 inactivation, as CDK4 depletion produced results similar to control knockdown cells (Figure 5A–D). In addition, pharmacologic inhibition of CDK4 using PD-0332991 increased resistance of these cells to HU (Figure 5F and G). These results indicate that CCND1, but not CDK4, is required for resistance of MCL cells to DNA replication inhibitors.

Effects of CCND1 depletion on the cell cycle of MCL cells

Because CCND1 primarily regulates cell-cycle progression through CDK4, increased chemosensitization in MCL cells after CCND1 depletion led us to examine the effect of loss of CCND1 on the cell cycle. Control or CCND1-depleted UPN-1 and JEKO-1 cells were labeled with bromodeoxyuridine (BrdU) and propidium iodide (PI), and the cell cycle was analyzed by flow cytometry. The CDK4 inhibitor PD-0332991 was also used in parallel experiments to compare with the effects of CCND1 depletion. Silencing of CCND1 in both UPN-1 and JEKO-1 cells was confirmed by qPCR (Figure 6A). While
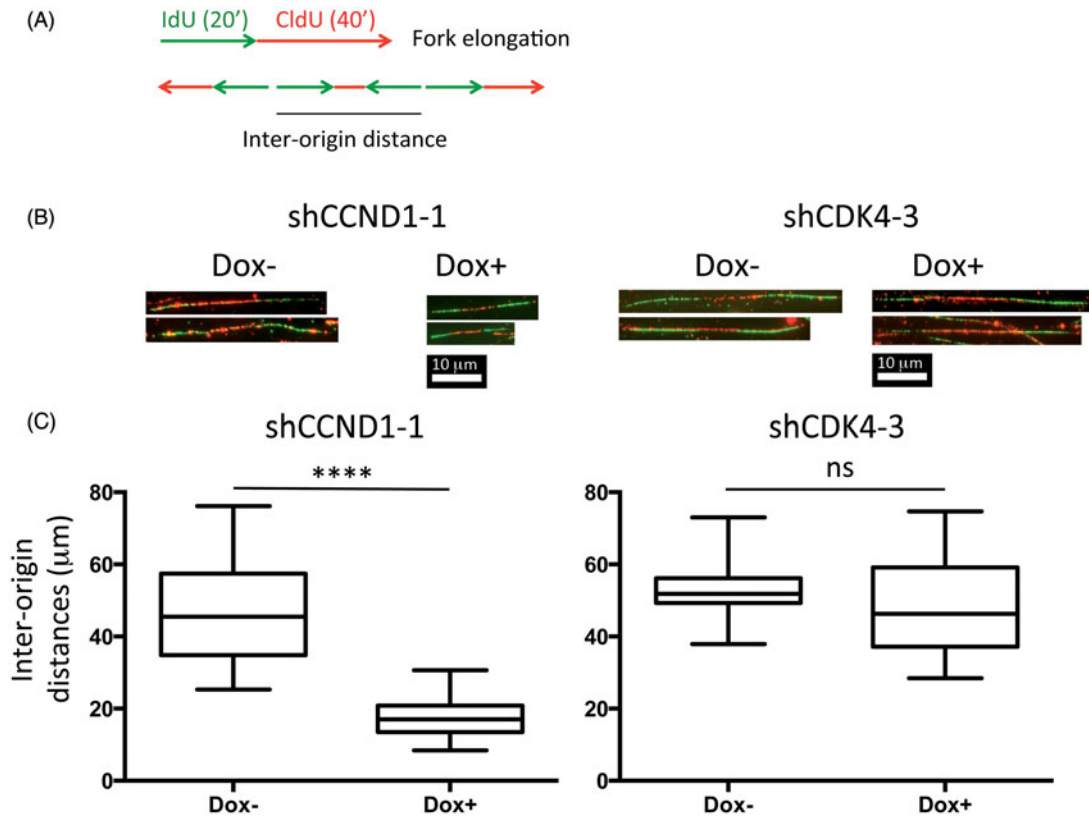
Figure 4. CCND1 depletion increases replication initiation frequency in UPN-1 cells. CCND1 or CDK4 shRNA-transduced UPN-1 cells were induced with Dox for four days and sequentially pulse-labeled with IdU and CldU, as illustrated in the diagram (A). (B) DNA fibers from labeled cells were spread on glass slides and incorporated IdU and CldU were detected by immunofluorescence. Shown are representative fluorescence images of labeled DNA fibers. (C) Inter-origin distances as illustrated in (A) and shown in (B) were measured in micrometers and quantified by boxplots, with bars contained in boxplots representing median values. Scale bars, 10 μm; ****$p < 0.0001$. ns: not significant. p values are from the two-tailed Mann-Whitney test for 100–200 scorable tracks on average per sample.

depletion of CCND1 or inhibition of CDK4 induced cell cycle arrest in both cell lines, more G1 arrest was observed in CDK4-inhibited cells (60–80%) than in CCND1-depleted cells (~50%) (Figure 6B and C). A strong induction of G1 arrest by CDK4 inhibition was consistent with the chemo-protective effect of PD-0332991, which prevented cells from entering the S phase where they were more sensitive to HU (Figure 5F and G). In contrast, weaker G1 arrest induced by CCND1 depletion sensitized cells to HU or cytarabine (Figure 5A–E). These data indicate a functional difference between CCND1 and CDK4 signaling with respect to chemosensitivity and suggest a CDK4-independent role of CCND1, which becomes essential when cells encounter replication inhibitors.

Discussion

The present study demonstrated that CCND1 is essential for the maintenance of established MCL tumor cell lines and uncovered a new role for CCND1 in preserving genomic stability during DNA replication in MCL cells. There are conflicting reports on the role of CCND1 in the survival of MCL cells. Klier et al. [30] showed that silencing of CCND1 in the MCL lines GRANTA-519, JEKO-1, and Z-138 for up to 7 days caused growth arrest but not cell death. In contrast, Weinstein et al. [31] detected apoptosis in the GRANTA-519 and JEKO-1 lines 8 days after CCND1 siRNA transfection. In agreement with the study of Weinstein et al.,[31] our data indicate that CCND1 is essential in JEKO-1 (and UPN-1) cells. Apoptosis in JEKO-1 cells after CCND1 depletion was detected both in the Weinstein et al. [31] and the present study but not in the study by Klier et al.[30] This discrepancy is probably due to the use of more sensitive apoptotic markers like Annexin V by Weinstein et al. [31] or active caspase 3/cleaved PARP 1 in our study, whereas Klier et al. [30] assessed apoptosis by quantifying the sub-diploid populations.
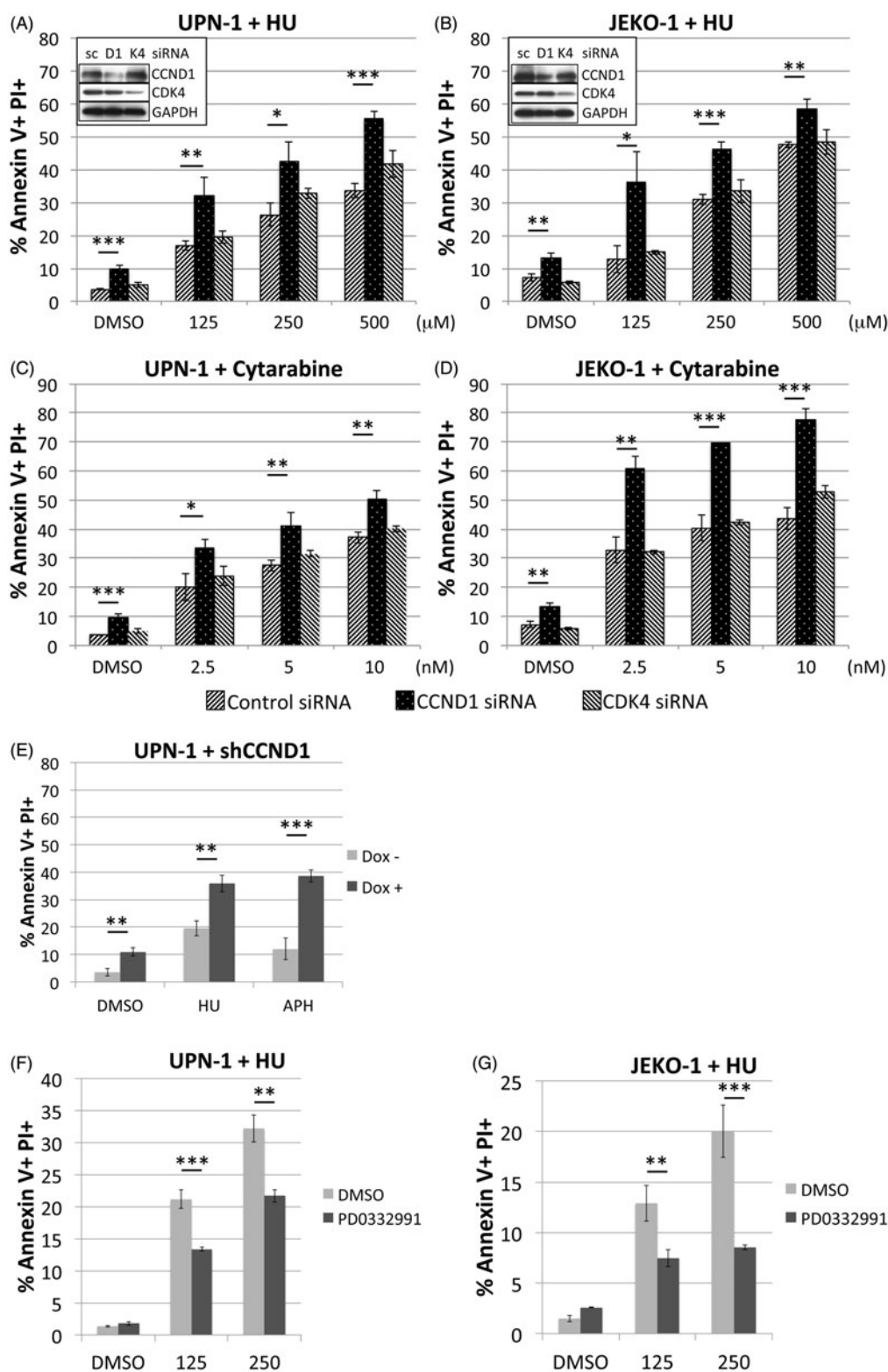
Figure 5. CCND1 depletion sensitizes MCL cells to DNA replication inhibitors. (A–D) MCL lines were transfected with siRNA against CCND1 (D1) or CDK4 (K4) or a control siRNA on day zero and again on day two (see Materials and Methods). On day three, 1.5×10^5 live cells were counted and re-plated, followed by addition of HU (A and B) or cytarabine (C and D) at indicated doses. Apoptosis was assessed by Annexin V/PI staining on day five. Shown are percentages of Annexin V+/PI+ cells. Inset, confirmation of protein knockdown three days after siRNA transfection by immunoblotting with indicated antibodies. (E) CCND1 shRNA sensitizes UPN-1 cells to DNA replication inhibitors. CCND1 shRNA-transduced UPN-1 cells were induced with Dox for four days followed by treatment with HU (0.25 mM) or aphidicolin (APH, 1000 nM) for two days and analyzed for apoptosis by Annexin V/PI staining. (F and G) CDK4 inhibition increases chemoresistance in MCL cells to HU. UPN-1 or JEKO-1 cells were treated with indicated doses of HU in combination with DMSO or 250 nM of PD-0332991 for 2 days and analyzed for apoptosis by Annexin V/PI staining. Bar graphs show means of three independent experiments. Error bars, SD. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$ by a two-tailed Student s t-test.
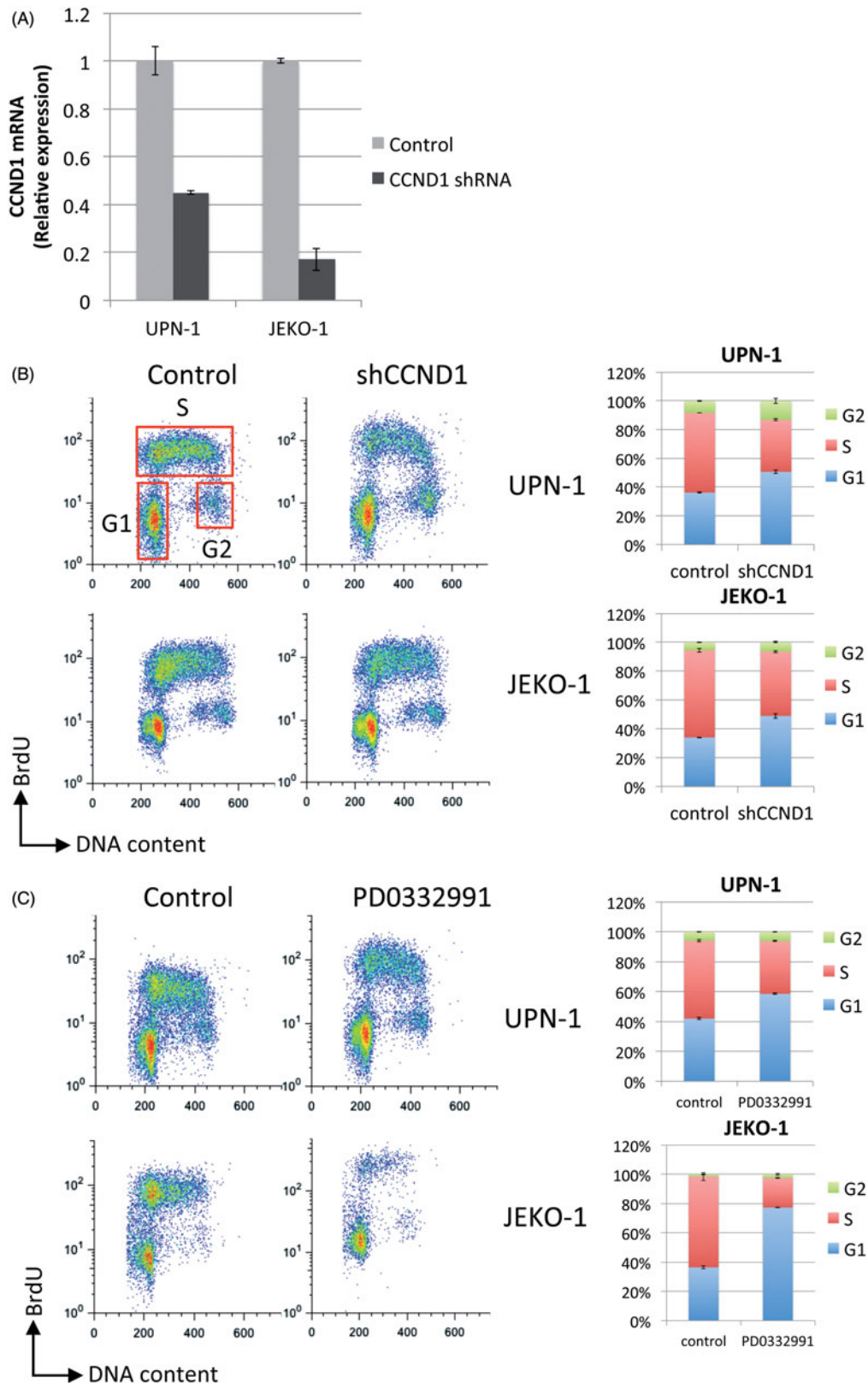
Figure 6. Effects of CCND1 depletion on the cell cycle. (A) Verification of CCND1 knockdown. Control or CCND1-depleted UPN-1 or JEKO-1 cells were analyzed for relative CCND1 mRNA expression by qPCR after 4 days of shRNA induction. Shown are the means of three independent experiments. Error bars, SD. (B) Cell cycle profiles of MCL cells after CCND1 depletion. Four days after CCND1 depletion, cells were BrdU labeled for 30 min followed by FACS analysis. Shown are representative FACS plots of control or CCND1 depleted UPN-1 or JEKO-1 cells. Stacked column graphs show percentages of cells in each cell cycle phase, as defined by the drawn gates. Values are the means from three independent experiments. Error bars, SD. (C) Cell cycle profiles of MCL cells after CDK4 inhibition. Indicated cells were treated with PD0332991 (250 nM) for 4 days and were labeled with BrdU as described in (B). Shown are representative FACS plots of control or treated UPN-1 or JEKO-1 cells. Stacked column graphs were generated as decribed in (B). Values are the means from three independent experiments. Error bars, SD.

We found that CCND1 depletion resulted in increased origin firing, leading to replication stress and DNA DSB. However, it is unclear how CCND1 plays a protective role in preventing DNA damage during replication in MCL cells. In a comprehensive proteomics study of CCND1-overexpressing human cancers, including one MCL line (Granta-519), Jirawatnotai et al. [32] demonstrated direct binding between CCND1 and the DNA repair protein RAD51 in the context of radiation-induced DNA damage. As RAD51 can also be recruited to the stalled replication forks independently of DNA breakage,[33,34] it would be of future interest to investigate whether CCND1 and RAD51 interaction is required to maintain genome stability during unperturbed replication in MCL.

Because CCND1 depletion is toxic in the MCL lines used in the present study, detailed studies of its function require a conditional knockdown system. By generating Dox-inducible knockdown cell lines in UPN-1 cells, we uncovered a previously unrecognized role for CCND1 in preserving genomic stability during DNA replication. This genome protective function of CCND1 is thus relevant to the resistance of MCL to chemotherapeutic agents. These data were, however, limited to the inducible knockdown system generated in UPN-1 cells. Multiple attempts to generate inducible CCND1 shRNA in JEKO-1 cells have been proven technically challenging, likely due to the inherent leakiness of the tetracycline inducible system in this cell line. Nonetheless, the roles of CCND1 in promoting survival and chemoresistance in JEKO-1 cells reported in this study are consistent with previous studies for this cell line.[21,31]

Aberrant CCND1 signaling is considered central to MCL pathogenesis and major efforts to target this pathway have been directed to its catalytic partner CDK4 using the highly specific inhibitor palbociclib (PD-0332991). Despite being a potent inhibitor of CDK4-RB signaling that can efficiently suppress tumor growth both *in vitro* and *in vivo*,[13–15] palbociclib induces little or very modest cell death in MCL, as demonstrated by others [15] and the present study. Our data indicate that the survival and chemoresistance of established human MCL lines are more dependent on CCND1 than on CDK4, suggesting CCND1 as a valid target in MCL. However, these cell line results were confirmed by only limited data on primary MCL cells (see Figure 2A) due to technical challenges in genetic manipulation of primary cells. Currently, there are no small-molecule inhibitors of CCND1 under clinical development, but pharmacologic agents that indirectly reduce CCND1 levels have been reported (see review in [35]). These agents will be helpful to confirm our cell line observations in primary MCL cells. Of interest, recent MCL treatment advances using ibrutinib, a Bruton's tyrosine kinase inhibitor, have produced durable responses in MCL patients.[36] Further studies by Ma et al. [37] to characterize MCL cells with different sensitivity to ibrutinib showed that this drug can down-regulate CCND1 levels in certain MCL cell lines. Our findings on the essential role of CCND1 in the survival and maintaining genomic stability of MCL cells are therefore highly relevant to future efforts to improve the efficacy of this emerging therapy.

In summary, this study has demonstrated a role for CCND1 in the survival and chemoresistance of MCL cells. CCND1 plays these essential roles likely through involvement in DNA replication to minimize DNA damage. These findings, thus, have important implications for understanding and treating the chemoresistance of aggressive MCL.

Acknowledgements

References

[1]   Jares P, Colomer D, Campo E. Genetic and molecular pathogenesis of mantle cell lymphoma: perspectives for new targeted therapeutics. Nat Rev Cancer. 2007;7:750–762.

[2]   Perez-Galan P, Dreyling M, Wiestner A. Mantle cell lymphoma: biology, pathogenesis, and the molecular basis of treatment in the genomic era. Blood. 2011;117:26–38.

[3]   Raffeld M, Jaffe ES. bcl-1, t(11;14), and mantle cell-derived lymphomas. Blood. 1991;78:259–263.

[4]   Sherr CJ. Mammalian G1 cyclins. Cell. 1993;73:1059–1065.

[5]   Hatakeyama M, Brill JA, Fink GR, et al. Collaboration of G1 cyclins in the functional inactivation of the retinoblastoma protein. Genes Dev. 1994;8: 1759–1771.

[6]   Calbo J, Parreno M, Sotillo E, et al. G1 cyclin/cyclin-dependent kinase-coordinated phosphorylation of endogenous pocket proteins differentially regulates their interactions with E2F4 and E2F1 and gene expression. J Biol Chem. 2002;277:50263–50274.

[7] Sherr CJ, Roberts JM. CDK inhibitors: positive and negative regulators of G1-phase progression. Genes Dev. 1999;13:1501–1512.

[8] Wlodarska I, Dierickx D, Vanhentenrijk V, et al. Translocations targeting CCND2, CCND3, and MYCN do occur in t(11;14)-negative mantle cell lymphomas. Blood. 2008;111:5683–5690.

[9] Salaverria I, Royo C, Carvajal-Cuenca A, et al. CCND2 rearrangements are the most frequent genetic events in cyclin D1(-) mantle cell lymphoma. Blood. 2013;121:1394–1402.

[10] Sonoki T, Harder L, Horsman DE, et al. Cyclin D3 is a target gene of t(6;14)(p21.1;q32.3) of mature B-cell malignancies. Blood. 2001;98:2837–2844.

[11] Kouroukis CT, Belch A, Crump M, et al. Flavopiridol in untreated or relapsed mantle-cell lymphoma: results of a phase II study of the National Cancer Institute of Canada Clinical Trials Group. J Clin Oncol. 2003;21:1740–1745.

[12] Lin TS, Blum KA, Fischer DB, et al. Flavopiridol, fludarabine, and rituximab in mantle cell lymphoma and indolent B-cell lymphoproliferative disorders. J Clin Oncol. 2010;28:418–423.

[13] Fry DW, Harvey PJ, Keller PR, et al. Specific inhibition of cyclin-dependent kinase 4/6 by PD 0332991 and associated antitumor activity in human tumor xenografts. Mol Cancer Ther. 2004;3:1427–1438.

[14] Leonard JP, LaCasce AS, Smith MR, et al. Selective CDK4/6 inhibition with tumor responses by PD0332991 in patients with mantle cell lymphoma. Blood. 2012;119:4597–4607.

[15] Marzec M, Kasprzycka M, Lai R, et al. Mantle cell lymphoma cells express predominantly cyclin D1a isoform and are highly sensitive to selective inhibition of CDK4 kinase activity. Blood. 2006;108:1744–1750.

[16] Kornmann M, Danenberg KD, Arber N, et al. Inhibition of cyclin D1 expression in human pancreatic cancer cells is associated with increased chemosensitivity and decreased expression of multiple chemoresistance genes. Cancer Res. 1999;59:3505–3511.

[17] Biliran H, Jr.,Wang Y, Banerjee S, et al. Overexpression of cyclin D1 promotes tumor cell growth and confers resistance to cisplatin-mediated apoptosis in an elastase-myc transgene-expressing pancreatic tumor cell line. Clin Cancer Res. 2005;11:6075–6086.

[18] Noel EE, Yeste-Velasco M, Mao X, et al. The association of CCND1 overexpression and cisplatin resistance in testicular germ cell tumors and other cancers. Am J Pathol. 2010;176:2607–2615.

[19] Hashimoto T, Yanaihara N, Okamoto A, et al. Cyclin D1 predicts the prognosis of advanced serous ovarian cancer. Exp Ther Med. 2011;2:213–219.

[20] Rosenwald A, Wright G, Wiestner A, et al. The proliferation gene expression signature is a quantitative integrator of oncogenic events that predicts survival in mantle cell lymphoma. Cancer Cell. 2003;3:185–197.

[21] Tiemann K, Alluin JV, Honegger A, et al. Small interfering RNAs targeting cyclin D1 and cyclin D2 enhance the cytotoxicity of chemotherapeutic agents in mantle cell lymphoma cell lines. Leuk Lymphoma. 2011;52:2148–2154.

[22] M'Kacher R, Farace F, Bennaceur-Griscelli A, et al. Blastoid mantle cell lymphoma: evidence for nonrandom cytogenetic abnormalities additional to t(11;14) and generation of a mouse model. Cancer Genet Cytogenet. 2003;143:32–38.

[23] Amin HM, McDonnell TJ, Medeiros LJ, et al. Characterization of 4 mantle cell lymphoma cell lines. Arch Pathol Lab Med. 2003;127:424–431.

[24] Ngo VN, Davis RE, Lamy L, et al. A loss-of-function RNA interference screen for molecular targets in cancer. Nature. 2006;441:106–110.

[25] Frum RA, Deb S, Deb SP. Use of the DNA fiber spreading technique to detect the effects of mutant p53 on DNA replication. Methods Mol Biol. 2013;962:147–155.

[26] Pinyol M, Bea S, Pla L, et al. Inactivation of RB1 in mantle-cell lymphoma detected by nonsense-mediated mRNA decay pathway inhibition and microarray analysis. Blood. 2007;109:5422–5429.

[27] Rogakou EP, Pilch DR, Orr AH, et al. DNA double-stranded breaks induce histone H2AX phosphorylation on serine 139. J Biol Chem. 1998;273:5858–5868.

[28] Fenech M. Cytokinesis-block micronucleus cytome assay. Nat Protoc. 2007;2:1084–1104.

[29] Osborn AJ, Elledge SJ, Zou L. Checking on the fork: the DNA-replication stress-response pathway. Trends Cell Biol. 2002;12:509–516.

[30] Klier M, Anastasov N, Hermann A, et al. Specific lentiviral shRNA-mediated knockdown of cyclin D1 in mantle cell lymphoma has minimal effects on cell survival and reveals a regulatory circuit with cyclin D2. Leukemia. 2008;22:2097–2105.

[31] Weinstein S, Emmanuel R, Jacobi AM, et al. RNA inhibition highlights cyclin D1 as a potential therapeutic target for mantle cell lymphoma. PLoS One. 2012;7:e43343

[32] Jirawatnotai S, Hu Y, Michowski W, et al. A function for cyclin D1 in DNA repair uncovered by protein interactome analyses in human cancers. Nature. 2011;474:230–234.

[33] Petermann E, Orta ML, Issaeva N, et al. Hydroxyurea-stalled replication forks become progressively inactivated and require two different RAD51-mediated pathways for restart and repair. Mol Cell. 2010;37:492–502.

[34] Hashimoto Y, Ray Chaudhuri A, Lopes M, et al. Rad51 protects nascent DNA from Mre11-dependent degradation and promotes continuous DNA synthesis. Nat Struct Mol Biol. 2010;17:1305–1311.

[35] Alao JP. The regulation of cyclin D1 degradation: roles in cancer development and the potential for therapeutic invention. Mol Cancer. 2007;6:24

[36] Wang ML, Rule S, Martin P, et al. Targeting BTK with ibrutinib in relapsed or refractory mantle-cell lymphoma. N Engl J Med. 2013;369:507–516.

[37] Ma J, Lu P, Guo A, et al. Characterization of ibrutinib-sensitive and -resistant mantle lymphoma cells. Br J Haematol. 2014;166:849–861.